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Antagonistic characterization of marine microalgae surface associated bacterium *Staphylococcus albus* SBU1 against selected human pathogens

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Abstract : Surface associated marine bacteria often produce secondary metabolites with antagonistic activities. We have isolated surface associated antagonistic bacteria from marine microalgae *Tetraselmis suecica* (Kylin). The antagonistic activity was screened against selected human pathogens. The potential isolate was identified as *Staphylococcus albus* SBU1. The antagonistic capability was evaluated at different physico-chemical parameters. Among the pathogens tested, highest antibacterial activity of 13 mm was noticed against *Salmonella* sp and 12 mm against *Bacillus subtilis* when the isolate incubated at 35^{o}C at the pH of 7 respectively. Column chromatography technique was adopted to purify the ethyl acetate crude extract. Each fraction was subjected to perform thin layer chromatography to calculate the retention factors (Rf) and to check the purity. The Rf value of fraction 1 and 2 were 0.65 and 61 respectively. All the fractions were adapted for to antibacterial tendency against test pathogens. A total of five fractions were analyzed, amusingly fraction 1 showed maximum activity (10 ± 0.70 mm) against *Streptococcus pyogens*. The current research specify that marine micro algae surface associated bacteria have a wide array of biosynthetic capabilities for the production of novel and unique secondary metabolites to treat infectious diseases.
Key words: Marine microalgae, surface bacteria, antagonism, human pathogens.

Introduction

Interestingly the world's oceans cover 70% of the earth planet's surface and living creatures on earth originates from sea. The marine environment harbors vast number of macro and microorganisms. These organisms have developed unique and unusual metabolic abilities to ensure their survival in diverse and antagonistic habitats. As a result the marine organisms have the adaptation in such a way in the biosynthesis of wide array of secondary metabolites with specific bioactivities [1]. The isolation and characterization of novel bioactive secondary metabolites from marine microorganisms have a clinical and pharmaceutical potential for future drug discovery to treat infectious diseases. Every year most of the infectious microbes are resistance to current clinical medicine [2]. Worldwide antimicrobial resistance is documented as one of the greatest threats to human health [3]. New drug therapies have extended human life span and also improved the quality of their life. In this regard, human society has become more and more dependent upon the availability of eco-friendly and efficacious pharmaceutical products. Currently, more than 50% of the marketed pharmaceutical products are either extracted from natural sources or produced by synthesis using natural products as starting materials. The marine environment turn into a focus of natural products drug discovery research because of its relatively unexplored and untapped biodiversity compared to terrestrial counter parts. So the researchers and scientists are

focusing marine resources to extract unique bioactive metabolites and its led component for the multi – drug resistant infectious organisms. Nowadays researchers are demonstrating the enormous drug discovery potential of microorganisms isolated from this adverse resource. Amusingly marine microorganisms constitute a fruitful source of structurally different novel bioactive metabolites [4]. Marine microbial natural products are exceptionally interesting high-value ingredients for applications in the pharmaceutical industry, due to their broad spectrum of bioactivities such as anti-tumor, anti-proliferative, photo-protective, antibiotic and anti-infective activities [5]. In recent years researchers focus on microorganism associated with the surface of marine organisms have been major targets for the discovery of new bioactive metabolites. Recently it was evident that many of the bioactive compounds previously isolated from marine macro organisms are in fact, metabolic products of associated microorganisms [6, 7]. In this regard the present study has been focused on surface associated bacteria from marine microalgae for their antibacterial potential against infectious human pathogens.

Materials and Methods

Marine microalgae

Marine microalgae *Tetraselmis suecica* (Kylin) was collected from Centre for Marine Fisheries Research Institute (CMFRI) Tuticorin, Tamilnadu, India in a sterile screw cap tube and kept in a thermo control box then brought to our laboratory. The microalgae were sub-cultured to get an auxenic culture for the present microalgae epiphytic bacterial secondary metabolites study.

Isolation of surface associated bacteria

Exponential phase microalgae culture (1ml) sample was taken and subjected to serial dilution up to 10^{-5} dilutions. The aliquots are plated in Zobell marine agar (ZMA) plates by pour plate method. The inoculated plates were incubated at 37^{o}C for 24 hours in an inverted position. Morphologically contradictory well isolated colonies were erratically selected and stored at 4^{o}C for further antagonistic screening and strain identification studies.

Human pathogens

Human bacterial pathogens both Gram negative viz., *Vibrio cholerae, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Salmonella* sp., *Proteus* sp. and Gram positive viz., *Streptococcus pyogens, Staphylococcus aureus, Bacillus megaterium* and *Bacillus subtilis,* were collected from Kanyakumari medical college and hospital (KMCH), Kanyakumari District, Tamilnadu, India for the present antibacterial susceptibility study.

Primary screening of antagonistic bacteria

The antagonistic activity (AGA) was deliberated by following standard cross streak assay method [8]. Single ribbon streak with the size of 4 – 6 mm diameter of the isolated bacteria were streaked on the surface of modified nutrient agar (MNA) plates and incubated at 37^{o}C for 48 h. The overnight pathogenic bacterial culture was streaked at right angles to the original ribbon streak of epiphytic bacteria and incubated at 37^{0}C for 24h. Triplicates were maintained for each isolates to evaluate the antagonistic activity. The zone of inhibition was measured from the edge of the ribbon streak to test bacteria and recorded in mm in diameter. A control plate was also maintained without inoculating epiphytic isolate to appraise the normal growth of test bacteria. All the bacterial isolates were subjected to evaluate for the antagonistic activity. The isolates which showed exceptional and broad spectrum activity were taken for further strain identification and mass scale production of bioactive compounds.

Identification of isolate

The bacteria which showed exceptional and broad spectrum activity was considered and subjected to streak plate technique to get pure culture. The potential candidate isolate was identified based on the morphological, physiological and biochemical characteristics and compared with Bergey's Manual of Determinative Bacteriology [9].

Mass cultivation of potential isolate

The potential candidate epiphytic bacterial strain of *Staphylococcus albus* SBU1 showing higher promising antagonistic activity was selected for mass scale production of antimicrobial metabolites. Single loop full culture of chosen bacterial strain was inoculated into 100ml of Zobel Marine broth (ZMB) and incubated in orbital shaking incubator (NEOLAB) for 24h. Ten milliliter of inoculum was transfer into 2000 ml conical flask containing 1000 ml of Zobel Marine broth with different culture parameter viz., pH (3, 5, 7, 9 and 11), and temperature (25, 30 and 35^{o}C) were optimized independently. The mass cultivation flask was incubated for 48 - 72h with constant shaking.

Extraction of secondary metabolites

Mass cultivated fermented (MCF) broth was mixed with equal volume of ethyl acetate (1:0.5 ratio) in a separating funnel. This mixture was vigorously shaken to extract the bioactive principle [10]. The mixture was kept in an undisturbed condition on the burette stand for 15min. The inferior aqueous phase was collected in new beaker. The superior organic solvent phase was collected in separate beaker to get crude secondary metabolites. This procedure was repeated three times to get complete extraction of bioactive metabolites. Ultimately the crude metabolite was concentrated in a rotary vacuum evaporator (Rotary Vacuum Evapor, Model: SSI-62) at 40^{o}C for 24 – 48h to obtain dry powder. This crude bioactive metabolite was used for further secondary antagonistic studies against human pathogens.

Antibacterial assay

Antibacterial activity was determined against the chosen clinical pathogens using paper disk assay (PDA) method [11]. Whatman No.1 filter paper disk of 6mm diameter was cut and sterilized by autoclaving. The sterile disk was impregnated with different bacterial extracts (50µl/per disk). Control disk also maintained for each bacterial extract by impregnate ethyl acetate alone. Muller Hinton Agar (MHA) plates were prepared and overnight broth culture (1.2×10^{8} cfu/ml) of test pathogens were inoculated uniformly using sterile cotton swab. The impregnated disks were placed on the plates using sterile forceps suitably spaced at equal distance. Triplicates were maintained for each test pathogen. The plates were incubated at 37°C for 24h. The zone of inhibition appearing around the discs were measured and expressed in mm in diameter.

Characterization of bacterial secondary metabolites

Column Chromatography (CC) of bioactive metabolites

The crude extract was dissolved in an appropriate volume of ethanol and used for further purification by the standard methods [12]. The extract was purified by silica gel column chromatography (SGCC) using silica gel (60 – 120 mesh) as stationary phase and ethanol: water (1:1 ratio v/v) as solvent phase. The column (35 X10 mm) was cleaned and packed with silica gel with sterile distilled water. The secondary metabolites were partially purified by elution with (10% stepwise) 0–100% by volume of ethanol in water through a silica gel column. The fractions were collected based on the elution at particular period of interval.

Thin Layer Chromatography (TLC) of bioactive metabolites

Each fraction was analyzed by thin layer chromatography (TLC) using pre-coated silica gel plates of 0.25 mm thickness (Merck, India) to identify the fraction that contained bioactive metabolites. To develop a chromatogram, Ethanol, water and chloroform were used in a volume ratio of 90:25:4. The eluted spot in the plate was visualized in the iodine vapour chamber and the Rf value of partially purified bioactive metabolite was calculated by the following formula.

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Chemicals and culture media

All chemicals and culture media ingredients were acquired from Hi media Laboratories Private Limited, (Mumbai, India) used to carry out the present investigation.

Data analysis

All data were statistically analyzed through TWO way ANOVA using MINITAB software. The means for different parameters were separated by applying least significant difference (LSD) test at 0.05 % level of probability to know their significance status.

Results and Discussion

Table 1 Preliminary screening of antagonistic surface associated bacteria by cross streak assay method.

Bacterial isolates	Human pathogens									
	Vibrio cholerae	Klebsiella pneumoniae	Escherichia coli	Pseudomonas aeruginosa	Salmonella sp	Proteus sp	Streptococcus pyogens	Staphylococcus aureus	Bacillus megaterium	Bacillus subtilis
SBU1	++	+++	++	++	+++	++	+	++	+++	+++
SBU2	++	++	++	+	++	++	++	+++	+++	++
SBU3	+	++	+++	+	+	+	+	++	++	++
SBU4	++	+	++	++	+	+	+	+	++	++
SBU5	++	+	+	-	+	+	++	++	++	++
SBU 6	-	-	-	-	-	-	-		+	+
SBU 7	+	+	+	+	+	+	+	+	-	-
SBU 8	-	-	-	-	-	-	-	-	-	-
SBU 9	-	+	+	+	+	+	++	++	+	+
SBU 10	+	-	-	-	+	+	++	++	+	+
SBU 11	-	-	-	-	-	-	+	+	+	+
SBU 12	+	+	+	+	+	+	-	-	-	-
SBU 13	-	-	-	-	-	-	-	-	-	
SBU 14	+	+	+	+	++	++	-	-	-	-
SBU 15	-	-	-	-	-	-	-	-	-	
SBU 16	-	-	-	-	-	-	++	++	+++	+++
SBU 17	+	+	+	+	+	+	++	++	++	++
SBU 18	-	+	+	+	+	+	++	++	++	++
SBU 19	+	+	+	+	+	+	-	-	-	-
SBU 20	-	-	-	-	-	-	-	-	-	-

Indication: +++ 10 - 15 mm; ++ 5-10 mm; +>5mm; - no activity

Marine habitat provides different biosynthetic conditions to microorganisms where they live in it. Most of the marine microbes generally live in symbiotic association with macro organisms. A recent trend in marine microbial natural products chemistry is the study of symbiosis to the partner and competitor to the other microbes [13]. Marine microalgae surface associated bacteria are unexplored group of microorganisms and antimicrobial characterization of potential candidate producers are still scanty. Surface associated bacteria were isolated from marine microalgae *Tetraselmis suecica* by standard pour plate technique. Morphologically dissimilar isolates were selected and re-streaked on Zobel marine agar to obtain pure culture. The primary screening of antagonistic bacteria was performed by standard cross streak assay method. Totally twenty surface associated bacteria have been isolated and subjected to screening for the antagonistic efficacy against chosen human pathogens. The intensity of antagonistic efficacy of isolates was assessed and is portrayed in Table 1. In the present investigation more than 80 % of isolates exhibit antagonistic activity against at least one of the tested human pathogens. These surface associated bacteria can produce secondary metabolites which inhibit the settlement of microbial competitors. Therefore surface associated bacteria are attracting significant attention as a source of innovative natural products [14]. The isolate which had broad spectrum and high antagonistic activity against tested human pathogens has been selected for further characterization studies. The marine microalgae associated bacterial strain which showed prominent and broad spectrum activity were subjected to species level identification. The colony morphology and cell wall type of our isolates belonged to gram positive cocci bacteria. The results were depicted in Table 2. The potential candidate isolate was identified based on the

biochemical characterization and named as *Staphylococcus albus* SBU1. Further the isolate was subjected for mass scale production of secondary metabolites.

Table 2 Biochemical characteristics of surface associated bacteria *Staphylococus albus* SBU1

Biochemical characteristics	Test results
Gram's staining	G +ve cocci
Motility test	Non-motile
Indole test	-
MR test	+
VP test	+
Citrate test	-
Urease test	+
Oxidase test	-
Nitrate test	+
H_2S production	slightly
Glucose	+

"+" denotes positive reactions; "-" denotes negative reactions

The most capable potential isolate of *S. albus* SBU1 was adopted separately for further mass scale production of secondary metabolites in different culture parameters under experimental condition. Bioactive compound extracted from the isolate was studied at different physical parameter v*iz.*, temperature and pH. The antibacterial efficacy of *S. albus* SBU1 crude ethyl acetate extract was adopted by disc diffusion method against human pathogens. The antibacterial efficacy at various temperatures and pH is portrayed in Graph 1 & Graph 2 respectively. Highest antibacterial efficacy was observed against *Salmonella* sp. (13mm) followed by *Bacillus subtilis* (12 mm) when the epiphytic bacteria incubated at 35^{o}C. When the media adjusted at the pH of 7 maximum antibacterial activity (12mm) was noticed against *Pseudomonas aeruginosa.* Moreover highest activity (12mm) was noticed against *Proteus* sp. when the culture media adjusted at the pH of 9. Antimicrobial activity data obtained from culture supernatant of epiphytic bacterial extract against human pathogens was analyzed, the values were significant ($P < 0.05$) between organism at pH 7 and highly significant at pH 7 and 9. Marwick *et al* [15] insisted that the pH level of the growth medium has a noticeable effect on secondary metabolic production. Marine bacteria grown in culture medium pH of nutrient medium may induce the biosynthetic pathway of secondary metabolite production.

Crude ethyl acetate extract of *S. albus* SBU1 was partially purified by silica gel column chromate graphy. In the present study partially purified fractions were collected based on the band separation at specified time intervals in separate beaker. Each fraction was checked for the purity of active metabolite by performing thin layer chromatography and calculates the retention factors (Rf) value. A single well separate band of the antimicrobial constituent was observed by TLC. Totally five fractions were collected by column chromatography. The Rf value of fraction 1 and fraction 2 was 65 and 0.61 respectively. The fluorescence colour of the spot was brownish. All the fractions were subjected to antibacterial tendency against human pathogens and the results are depicted in Table 3. Out of five fractions tested, fraction 1 exhibited antimicrobial activity against all the tested pathogens except *K. pneumoniae*. Fraction 2 showed antimicrobial activity against all the tested pathogens except *B.megaterium.* Amusingly fraction 1 showed maximum activity (10 ± 0.70 mm) against *Streptococcus pyogens*. Fraction 2 exhibited highest activity (10.2 ± 0.83 mm) against *Pseudomonas aeruginosa.* However no activity was noticed from fractions 3 to 5 against tested human pathogens. This found that there is no active principles present in these fractions. Partially purified marine bacterial secondary metabolites demonstrated antimicrobial activity against both gram positive and gram negative organisms. These results indicate that partially purified secondary metabolites have selective antibacterial response mechanisms against pathogens rather than the crude extract [16]. Therefore it is possible that marine microalgae associated bacteria used in this study, share similar defense mechanisms against human pathogens. The surface associated bacteria creating an ecological importance as well as biotechnological interest in marine natural product. Further, compound characterization studies under progress to pin point the precise component responsible for the actual antimicrobial activity.

Table 3 Antibacterial efficacy of column purified active fractions of *S.albus* SBU1 ethyl acetate extract

Antibacterial metabolites	Zone of inhibition in mm diameter										
	Control	Vibrio cholerae	Klebsiella pneumonia	Escherichia coli	Staphylococcus aureus	Bacillus megaterium	Pseudomonas aeruginosa	Bacillus subtilis	Salmonella sp.	Proteus sp.	Streptococcus pyogens
Fraction 1	-	9.4 ± 1.14	-	8.6 ± 0.54	8.6 ± 0.54	8.2 ± 0.83	8.4 ± 1.34	7.8 ± 0.83	8.8 ± 0.83	9.4 ± 1.14	10 ± 0.70
Fraction 2	-	9 ± 0.70	8.4 ± 0.54	8.8 ± 0.83	10 ± 1	-	10.2 ± 0.83	9.6 ± 1.14	9.4 ± 1.14	9.2 ± 0.44	9.6 ± 1.34
Fraction 3	-	-	-	-	-	-	-	-	-	-	-
Fraction 4	-	-	-	-	-	-	-	-	-	-	-
Fraction 5	-	-	-	-	-	-	-	-	-	-	-

"-" No activity ; Each value is the mean ± SD of three individual estimates

Conclusion

This study showed that surface associated bacteria *S. albus* SBU1 isolated from marine microalgae *Tetraselmis suecica* had broad spectrum of antagonistic activity against gram positive and gram negative human pathogens such as *Salmonella* sp and *Bacillus subtilis*. It is obvious that surface associated bacterial strain produces a novel compound which may be the substance responsible for actual antibacterial activity. Thus, surface associated bacteria of microalgae may perhaps yield an immense array of secondary metabolites with unique activities. This research will initiate a new platform to provide a novel metabolite to fight back against a number of human infectious pathogens.

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References

1. Ana M, Helena V, Susana S (2014) Marketed Marine Natural Products in the Pharmaceutical and Cosmeceutical Industries: Tips for Success. Marine Drugs 12(2): 1066-1101.
2. Brad S, Robert G, David G, John B, Helen WB, Scheld WM, Bartlett JG, John E (2008) The Epidemic of Antibiotic-Resistant Infections: A Call to Action for the Medical Community from the Infectious Diseases Society of America. Clin Infect Dis. 46 (2): 155-164.
3. Walker B, Barrett S, Polasky S, et al., (2009) Environment. Looming global-scale failures and missing institutions. Science 325:1345-6.
4. Waters AL, Hill RT, Place AR, Hamann MT (2010) The expanding role of marine microbes in pharmaceutical development. Curr. Opin. Biotechnol. 21:780–786.
5. Schumacher M, Kelkel M, Dicato M, Diederich M (2011) Gold from the sea: Marine compounds as inhibitors of the hallmarks of cancer. Biotechnol. Adv. 29:531–547.
6. Penesyan A, Jan T, Suhelen E (2011) Identification of the Antibacterial Compound Produced by the Marine Epiphytic Bacterium *Pseudovibrio* sp. D323 and Related Sponge-Associated Bacteria. Marine Drug 9(8): 1391-1402.
7. Penesyan A, Kjelleberg S, Egan S (2010) Development of novel drugs from marine surface associated microorganisms. Marine Drugs. 8:438–459.

8. Ellaiah P, Kalyan D, Rao VS, Rao BV (1996) Isolation and characterization of bioactive actinomycetes from marine sediments. Hindustan Antibiot. Bull. 38*:* 48–52.
9. Holt JG, Krieg NR, Sneathm PHA, Staley JT, Williams ST (1994) Bergey's Manual of Determinative Bacteriology, 9th edn. Baltimore, MD: Williams and Williams.
10. Krishnakumar *S*, (2005) Isolation and standardization of antimicrobial compounds from Sponge associated antagonistic actinomycetes. *Ph.D.*, Thesis, Manonmanium Sundaranar University, Tamilnadu, India.
11. El-Masry HA, Fahmy HH, Abdelwahed ASH (2000) Synthesis and Antimicrobial activity of some new benzimidazole derivatives. Molecules 5: 1429-1438.
12. Krishnakumar S, Dooslin Mercy Bai V (2014) Antagonistic characterization of marine microalgae epiphytic bacterium *Pseudomonas maltophilia* SU2 against selected clinical pathogens**.** Int J Pharm Bio Sci 5(4): (B) 954 – 964.
13. Nisha C, Sunil S, Jyoti R, Inamullah, Surabhi S (2013) Marine Organism: A Rich Source of Bioactive Metabolites. IJCPS 1(4): 273-280.
14. Horta A, Pinteus S, Alves C, Fino N, Silva J, Fernandez S, Rodrigues A, Pedrosa R (2014) Antioxidant and Antimicrobial Potential of the *Bifurcaria bifurcata* epiphytic bacteria. Mainer Drugs 12(3):1676-1689.
15. Marwick JD, Wright PC, Burgress JG (1999) Bioprocess intensification for production of novel marine bacterial antibiotics through bioreactor operation and design. Marine Bacteriol, 1: 495-507.
16. Madhava Charyulu E, Sekaran G, Suseela Rajakumar G, Gnanamani A (2009) Antibacterial activity of secondary metabolite from marine isolate, *Pseudomonas* sp. against gram positive and negative bacteria including MRSA. Indian J experimental biology, 47: 964-968.

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